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(71) Applicant (for all designated States except US): CHIRON CORPORATION [US/US]; 4560 Horton Street, Emeryville, CA 94608 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): CHU, Keting [US/US]; 2017 Easton Drive, Burlingame, CA 94010 (US). WANG, Changyu [CN/US]; 3101 Carlson Boulevard #4, El Cerrito, CA 94530 (US). YOSHIHARA, Corrine [US/US]; 4560 Horton Street, Emeryville, CA 94608 (US). DONNELLY, John, J. [US/US]; 4560 Horton Street, Emeryville, CA 94608 (US).

(74) Agents: ALEXANDER, Lisa; Chiron Corporation, 4560 Horton Street, Emeryville, CA 94608-2916 et al. (US).

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(54) Title: HUMAN ANTI-CD40 ANTIBODIES

(57) Abstract: Human antibodies capable of binding CD40 are disclosed, wherein the antibodies act as antagonists of CD40-directed activities of B cells, but have no or minimal ability to induce B cell proliferation. The antibodies are useful for treating diseases mediated by CD40-expressing cells, such as those diseases characterized by B cell activation, as well as cancer of B-cell lineage, including Non-Hodgkin's Lymphoma.

HUMAN ANTI-CD40 ANTIBODIES

BACKGROUND OF THE INVENTION

Field of the Invention

The invention relates to human antibodies capable of binding to CD40,
5 methods of using the antibodies, and treatment of antibody-mediated disease in humans.

Description of the Related Art

The CD40 antigen is a glycoprotein expressed on the cell surface of B cells and other cells, including dendritic cells. During B-cell differentiation, the molecule is first expressed on pre-B cells and then disappears from the cell surface when the B cell becomes
10 a plasma cell. Crosslinking of the CD40 molecules with anti-CD40 antibodies mediates a variety of effects on B cells. The CD40 antigen is known to be related to the human nerve growth factor (NGF) receptor and tumor necrosis factor- α (TNF- α) receptor, suggesting that CD40 is a receptor for a ligand with important functions in B-cell activation.

CD40 is a key element of immune responses. Engagement of CD40 on
15 antigen-presenting cells by its ligand, termed CD40L or CD154, causes production of cytokines and up-regulation of costimulatory molecules leading to efficient activation of T lymphocytes. Engagement of CD40 on B lymphocytes provides a costimulatory signal to the B cell that drives antibody production. Thus blocking of CD40 engagement and activation has the potential to suppress antibody and cell mediation immune responses.
20 Anti-CD40 antagonist antibodies could be used to treat autoimmune disease such as systemic lupus, psoriasis, multiple sclerosis, inflammatory bowel disease (Crohn's disease), and rheumatoid arthritis. Such antibodies could also be used to prevent rejection of organ and tissue grafts by suppressing autoimmune responses, to treat lymphomas by depriving malignant B lymphocytes of the activating signal provided by CD40, and to deliver toxins
25 to CD40-bearing cells in a specific manner.

Previously, mouse monoclonal antibodies such as 5D12 have been disclosed that bind to CD40 without providing an activating signal. These antibodies have the ability to inhibit immune responses *in vivo* and *in vitro*. However mouse antibodies cannot be used to treat human disease because they elicit human anti-mouse antibodies that hinder the effectiveness of the treatment. Therefore, there is a need in the art for antibodies of comparable specificity but composed of a human amino acid sequence.

BRIEF SUMMARY OF THE INVENTION

It is a primary object of this invention to provide a human monoclonal antibody capable of binding to a human CD40 antigen located on the surface of a human B cell, wherein the binding of the antibody to the CD40 antigen prevents the growth or differentiation of the B cell.

It is a further object of this invention to provide a method for preventing or treating an antibody-mediated disease in a patient, the method comprising administering to a patient in need of such treatment a therapeutically effective amount of a human monoclonal antibody capable of binding to a human CD40 antigen located on the surface of a CD40-bearing cell such as a human B cell or a human dendritic cell, wherein the binding of the antibody to the CD40 antigen prevents the growth or differentiation of the cell, in a pharmaceutically acceptable excipient.

It is another object of this invention to provide a method for preventing or treating an IgE-mediated disease such as an allergy in a patient, the method comprising administering to a patient in need of such treatment a therapeutically effective amount of a human monoclonal antibody capable of binding to a human CD40 antigen located on the surface of a human B cell, wherein the binding of the antibody to the CD40 antigen prevents the growth or differentiation of the B cell, in a pharmaceutically acceptable excipient.

It is yet another object of this invention to provide a method for preventing or treating an autoimmune disease in a patient, including an antibody-mediated disease, the method comprising administering to a patient in need of such treatment a therapeutically

effective amount of a human monoclonal antibody capable of binding to a human CD40 antigen located on the surface of a human B cell, wherein the binding of the antibody to the CD40 antigen prevents the growth or differentiation of the B cell, in a pharmaceutically acceptable excipient. Particular autoimmune diseases contemplated for treatment by this
5 method include systematic lupus erythematosus (SLE), primary biliary cirrhosis (PBC), and idiopathic thrombocytopenic purpura (ITP).

It is another object of the invention to provide a method of inhibiting growth of tumor cells, including Non-Hodgkins Lymphoma cells.

DETAILED DESCRIPTION OF THE INVENTION

10 Antibodies are constructed of several regions, a crucial region being the complementarity determining region, or CDR. The phrase "complementarity determining region" refers to amino acid sequences which together define the binding affinity and specificity of the natural Fv region of a native immunoglobulin binding site. *See, e.g.,* Chothia et al., *J. Mol. Biol.* 196:901-917 (1987); Kabat et al., U.S. Dept. of Health and
15 Human Services NIH Publication No. 91-3242 (1991). The phrase "constant region" refers to the portion of the antibody molecule that confers effector functions. In previous work directed towards producing non-immunogenic antibodies for use in therapy of human disease, mouse constant regions were substituted by human constant regions. The constant regions of the subject humanized antibodies were derived from human immunoglobulins.
20 However, these humanized antibodies still elicited an unwanted and potentially dangerous immune response in humans and there is a loss of affinity.

The human monoclonal anti-CD40 antibodies of the present invention address the shortcomings of prior art monoclonal antibodies. Accordingly, the human monoclonal antibodies of the invention are preferably produced using transgenic animals
25 that are engineered to contain human immunoglobulin loci. For example, WO 98/24893 discloses transgenic animals having a human Ig locus wherein the animals do not produce functional endogenous immunoglobulins due to the inactivation of endogenous heavy and

light chain loci. WO 91/10741 also discloses transgenic non-primate mammalian hosts capable of mounting an immune response to an immunogen, wherein the antibodies have primate constant and/or variable regions, and wherein the endogenous immunoglobulin-encoding loci are substituted or inactivated. WO 94/02602 discloses non-human 5 mammalian hosts having inactivated endogenous Ig loci and functional human Ig loci. U.S. Patent No. 5,939,598 discloses methods of making transgenic mice in which the mice lack endogenous heavy chains, and express an exogenous immunoglobulin locus comprising one or more xenogeneic constant regions.

Using a transgenic animal described above, an immune response can be 10 produced to a selected antigenic molecule, in this case CD40, and antibody-producing cells can be removed from the animal and used to produce hybridomas that secrete human monoclonal antibodies. Immunization protocols, adjuvants, and the like are known in the art, and are used in immunization of, for example, a transgenic mouse as described in WO 96/33735. The monoclonal antibodies can be tested for the ability to inhibit or neutralize 15 the biological activity or physiological effect of the corresponding protein.

As used herein, the term "antibody" refers to polyclonal antibodies, monoclonal antibodies, single-chain antibodies, and fragments thereof such as Fab, F(ab')₂, F_v, and other fragments which retain the antigen binding function of the parent antibody.

As used herein, the term "monoclonal antibody" refers to an antibody 20 composition having a homogeneous antibody population. The term is not limited regarding the species or source of the antibody, nor is it intended to be limited by the manner in which it is made. The term encompasses whole immunoglobulins as well as fragments such as Fab, F(ab')₂, F_v, and others which retain the antigen binding function of the antibody. Monoclonal antibodies of any mammalian species can be used in this invention. 25 In practice, however, the antibodies will typically be of rat or murine origin because of the availability of rat or murine cell lines for use in making the required hybrid cell lines or hybridomas to produce monoclonal antibodies.

As used herein, the term "single chain antibodies" refer to antibodies prepared by determining the binding domains (both heavy and light chains) of a binding antibody, and supplying a linking moiety which permits preservation of the binding function. This forms, in essence, a radically abbreviated antibody, having only that part of the variable domain necessary for binding to the antigen. Determination and construction of single chain antibodies are described in U.S. Pat. No. 4,946,778 to Ladner et al.

The term "CD40 antigen epitope" as used herein refers to a molecule which is capable of immunoreactivity with the anti-CD40 monoclonal antibodies of this invention, excluding the CD40 antigen itself. CD40 antigen epitopes may comprise proteins, protein fragments, peptides, carbohydrates, lipids, and other molecules, but for the purposes of the present invention are most commonly proteins, short oligopeptides, oligopeptide mimics (*i.e.*, organic compounds which mimic the antibody binding properties of the CD40 antigen), or combinations thereof. Suitable oligopeptide mimics are described, *inter alia*, in PCT application US91/04282.

The antibodies of the current invention are produced by transgenic mice bearing human immunoglobulin loci, and bind to a human CD40 antigen on the surface of a human cell, particularly a B cell. These antibodies may be polyclonal antibodies, monoclonal antibodies, single-chain antibodies, and fragments thereof.

Monoclonal antibodies are prepared as described in the Examples. Other antibodies of the invention may be prepared similarly using mice transgenic for human immunoglobulin loci or by other methods known in the art and/or described herein.

Polyclonal sera may be prepared by conventional methods. In general, a solution containing the CD40 antigen is first used to immunize a suitable animal, in the present invention a transgenic animal, preferably a mouse bearing human immunoglobulin loci. In a preferred embodiment, Sf9 cells expressing CD40 are used as the immunogen. Immunization can also be performed by mixing or emulsifying the antigen-containing solution in saline, preferably in an adjuvant such as Freund's complete adjuvant, and injecting the mixture or emulsion parenterally (generally subcutaneously or

intramuscularly). A dose of 50-200 µg/injection is typically sufficient. Immunization is generally boosted 2-6 weeks later with one or more injections of the protein in saline, preferably using Freund's incomplete adjuvant. One may alternatively generate antibodies by *in vitro* immunization using methods known in the art, which for the purposes of this 5 invention is considered equivalent to *in vivo* immunization.

Polyclonal antisera are obtained by bleeding the immunized animal into a glass or plastic container, incubating the blood at 25°C for one hour, followed by incubating at 4°C for 2-18 hours. The serum is recovered by centrifugation (e.g., 1,000 x g for 10 minutes). About 20-50 ml per bleed may be obtained from rabbits.

10 Monoclonal antibodies can be prepared using the method of Kohler and Milstein, *Nature* 256:495-96 (1975), or a modification thereof. Typically, a mouse is immunized as described above. However, rather than bleeding the animal to extract serum, the spleen (and optionally several large lymph nodes) are removed and dissociated into single cells. If desired, the spleen cells may be screened (after removal of nonspecifically 15 adherent cells) by applying a cell suspension to a plate or well coated with the protein antigen. B-cells expressing membrane-bound immunoglobulin specific for the antigen bind to the plate, and are not rinsed away with the rest of the suspension. Resulting B-cells, or all dissociated spleen cells, are then induced to fuse with myeloma cells to form hybridomas, and are cultured in a selective medium (e.g., hypoxanthine, aminopterin, 20 thymidine medium, "HAT"). The resulting hybridomas are plated by limiting dilution, and are assayed for the production of antibodies which bind specifically to the desired immunizing cell-surface antigen (and which do not bind to unrelated antigens). The selected MAb-secreting hybridomas are then cultured either *in vitro* (e.g., in tissue culture bottles or hollow fiber reactors), or *in vivo* (as ascites in mice).

25 As an alternative to use of hybridomas, antibody can be produced in a cell line such as a CHO cell line, as disclosed in U.S. Patent Nos. 5,545,403, 5,545,405, and 5,998,144, incorporated herein by reference. Briefly, the cell line is transfected with vectors capable of expressing a light chain and a heavy chain, respectively. By transfecting

the two proteins on separate vectors, chimeric antibodies can be produced. Another advantage is the correct glycosylation of the antibody.

Preferably, fully human antibodies to CD40 are obtained by immunizing transgenic mice. One such mouse is referred to as a Xenomouse, and is disclosed in U.S.

5 Patent Nos. 6,075,181; 6,091,001; and 6,114,598, all of which are incorporated herein by reference. To produce the antibodies disclosed herein, mice transgenic for the human IgG2 heavy chain locus and the human K light chain locus are immunized with Sf9 cells expressing human CD40. Mice can also be transgenic for other isotypes.

Production of the Sf9 (*Spodoptera frugiperda*) cells is disclosed in de Boer, 10 U.S. Patent No. 6,004,552, incorporated herein by reference. Briefly, sequences encoding human CD40 are recombined into a baculovirus using transfer vectors as described by de Boer. The plasmids are co-transfected with wild-type baculovirus DNA into Sf9 cells. Recombinant baculovirus-infected Sf9 cells are identified and clonally purified.

Mice are injected intraperitoneally (IP) at day 0 and day 14 with about 15 5×10^6 Sf9 cells expressing CD40. A final injection is made at least five weeks later, and the spleen and thymus cells are removed and used for cell fusion. Cell fusion is carried out as described by de Boer. Hybridoma antibodies are screened as described in the Examples. Hybridomas are selected for further study based on their ability to inhibit proliferation of 20 human peripheral blood B cells induced by CD40 ligand (CD40L) and anti-IgM, and their ability to inhibit production of IgM by human peripheral blood B cells stimulated with anti-CD3-activated human peripheral blood T cells.

The relative binding properties of the hybridoma antibodies is examined by flow cytometry, as described in detail in the Examples. Briefly, the antibodies compared may exhibit differences in affinity despite their ability to recognize the same or closely 25 related epitopes.

To determine the polynucleotide sequences encoding the monoclonal antibodies, mRNA is prepared from the hybridomas and RT-PCR is performed on the

mRNAs using standard procedures. The PCR products are analyzed on gels, sequenced, and translated.

Preferred monoclonal antibodies prepared according to the invention, as well as fragments and chimeric forms thereof, will have antagonistic features that make 5 them suitable for a number of clinical applications, including treatment of autoimmune diseases, treatment of transplantation reactions and rejections, and as adjuvant therapies for gene therapies and protein therapies. Additional uses include treatment of any disease mediated by a CD40-expressing malignant cell, and use to treat diseases related to the proliferation, activation, or regulation of cells expressing CD40.

10 The invention also encompasses de-immunized antibodies, which can be produced as described in, for example, WO 98/52976, "Method for the Production of Non-Immunogenic Proteins," which is incorporated by reference herein. Also included within the scope of the claims are fusion proteins comprising a monoclonal antibody of the invention, or a fragment thereof, which fusion proteins can be synthesized or expressed 15 from corresponding polynucleotide vectors, as is known in the art.

The antibodies of the present invention can have sequence variations produced using methods described in, for example, Patent Publication Nos. EP 0 983 303 A1, WO 00/34317, and WO 98/52976, incorporated herein by reference. For example, it has been shown that sequences within the CDR can cause an antibody to bind to MHC 20 Class II and trigger an unwanted helper T cell response. A conservative substitution can allow the antibody to retain binding activity yet lose its ability to trigger an unwanted T cell response. Any such conservative or non-conservative substitutions can be made using art-recognized methods, and the resulting antibodies will fall within the scope of the invention.

Using only routine methods, one of skill in the art can construct plasmids 25 that will encode variants of the sequences of preferred antibodies. The variant antibodies can be routinely tested for antagonistic activity, affinity, specificity, and agonistic activity using methods described herein.

An antibody produced by any of the methods described above, or any other method not disclosed herein, will fall within the scope of the invention if it possesses at least one of the following biological activities: inhibition of immunoglobulin secretion by human peripheral B cells stimulated by T cells; inhibition of proliferation of human peripheral B cells stimulated by Jurkat T cells; and inhibition of proliferation of human peripheral B cells stimulated by CD40L-expressing cells. These assays can be performed as described in the Examples herein.

The antibodies will also exhibit a single site binding affinity (K_D) of at least 10^{-5} M, preferably at least 10^{-6} - 10^{-7} M, more preferably at least 10^{-8} M, and most preferably at least 10^{-9} M, such as 10^{-10} M, as measured using a standard assay such as Biacore, which is known in the art, in comparison with appropriate controls. Binding affinity of 10^{-11} M, 10^{-13} M, 10^{-15} M, 10^{-17} M and 10^{-19} M can also be achieved. These assays are automated, and allow the measurement of MAb specificity and cross-reactivity, which can also be assayed using standard techniques known in the art. Details of the Biacore assays are provided in Biacore's "BIAapplications handbook." Methods described in WO 01/27160 can be used to modulate the binding affinity.

If desired, the antibodies (whether polyclonal or monoclonal) may be labeled using conventional techniques. Suitable labels include fluorophores, chromophores, radioactive atoms (particularly ^{32}P and ^{125}I), electron-dense reagents, enzymes, and ligands having specific binding partners. Enzymes are typically detected by their activity. For example, horseradish peroxidase is usually detected by its ability to convert 3,3',5,5'-tetramethylbenzidine (TMB) to a blue pigment, quantifiable with a spectrophotometer. "Specific binding partner" refers to a protein capable of binding a ligand molecule with high specificity, as for example in the case of an antigen and a monoclonal antibody specific therefor. Other specific binding partners include biotin and avidin or streptavidin, IgG and protein A, and the numerous receptor-ligand couples known in the art. It should be understood that the above description is not meant to categorize the various labels into distinct classes, as the same label may serve in several different modes.

For example, ^{125}I may serve as a radioactive label or as an electron-dense reagent. HRP may serve as enzyme or as antigen for a MAb. Further, one may combine various labels for desired effect. For example, MAbs and avidin also require labels in the practice of this invention: thus, one might label a MAb with biotin, and detect its presence with avidin labeled with ^{125}I , or with an anti-biotin MAb labeled with HRP. Other permutations and possibilities will be readily apparent to those of ordinary skill in the art, and are considered as equivalents within the scope of the instant invention.

Antibodies for use in the invention can be produced using any suitable technique. For example, WO 01/27160 discloses a method of conferring donor CDR binding affinity onto an antibody acceptor variable region framework. The method can also be used to optimize the binding affinity of a variable region or an antibody, such as to enhance the binding affinity. Methods for producing humanized antibodies having one or more CDR's are disclosed in U.S. Patent No. 6,180,370. Methods of producing antibodies that have been optimized for administration to humans are disclosed in WO 00/34317, which describes the production of proteins that are rendered less immunogenic or non-immunogenic. U.S. Patent No. 5,514,548 discloses methods for selection of ligand binding proteins, such as antibodies, that bind with high affinity to a target ligand. U.S. Patent No. 5,877,397 disclosed transgenic non-human animals capable of producing heterologous antibodies. All of these patents and patent publications are incorporated herein by reference.

FORMULATIONS AND METHODS OF ADMINISTRATION

The antibodies of this invention are administered at a concentration that is therapeutically effective to prevent or treat antibody-mediated diseases such as allergies, SLE, PBC, ITP, multiple sclerosis, psoriasis, Crohn's disease, graft rejection, and B cell lymphoma. To accomplish this goal, the antibodies may be formulated using a variety of acceptable excipients known in the art. Typically, the antibodies are administered by injection, either intravenously or intraperitoneally. Methods to accomplish this

administration are known to those of ordinary skill in the art. It may also be possible to obtain compositions which may be topically or orally administered, or which may be capable of transmission across mucous membranes.

Before administration to patients, formulants may be added to the antibodies. A liquid formulation is preferred. For example, these formulants may include oils, polymers, vitamins, carbohydrates, amine acids, salts, buffers, albumin, surfactants, or bulking agents. Preferably carbohydrates include sugar or sugar alcohols such as mono-, di-, or polysaccharides, or water soluble glucans. The saccharides or glucans can include fructose, dextrose, lactose, glucose, mannose, sorbose, xylose, maltose, sucrose, dextran, pullulan, dextrin, alpha and beta cyclodextrin, soluble starch, hydroxethyl starch and carboxymethylcellulose, or mixtures thereof. Sucrose is most preferred. "Sugar alcohol" is defined as a C₄ to C₈ hydrocarbon having an -OH group and includes galactitol, inositol, mannitol, xylitol, sorbitol, glycerol, and arabitol. Mannitol is most preferred. These sugars or sugar alcohols mentioned above may be used individually or in combination. There is no fixed limit to amount used as long as the sugar or sugar alcohol is soluble in the aqueous preparation. Preferably, the sugar or sugar alcohol concentration is between 1.0 w/v % and 7.0 w/v %, more preferable between 2.0 and 6.0 w/v %. Preferably amino acids include levorotary (L) forms of carnitine, arginine, and betaine; however, other amino acids may be added. Preferred polymers include polyvinylpyrrolidone (PVP) with an average molecular weight between 2,000 and 3,000, or polyethylene glycol (PEG) with an average molecular weight between 3,000 and 5,000. It is also preferred to use a buffer in the composition to minimize pH changes in the solution before lyophilization or after reconstitution. Most any physiological buffer may be used, but titrate, phosphate, succinate, and glutamate buffers or mixtures thereof are preferred. Most preferred is a citrate buffer. Preferably, the concentration is from 0.01 to 0.3 molar. Surfactants that can be added to the formulation are shown in EP Nos. 270,799 and 268,110.

Additionally, antibodies can be chemically modified by covalent conjugation to a polymer to increase their circulating half-life, for example. Preferred

polymers, and methods to attach them to peptides, are shown in U.S. Pat. Nos. 4,766,106; 4,179,337; 4,495,285; and 4,609,546 which are all hereby incorporated by reference in their entireties. Preferred polymers are polyoxyethylated polyols and polyethylene glycol (PEG). PEG is soluble in water at room temperature and has the general formula: R(O--
5 CH₂--CH₂)_n O--R where R can be hydrogen, or a protective group such as an alkyl or alkanol group. Preferably, the protective group has between 1 and 8 carbons, more preferably it is methyl. The symbol n is a positive integer, preferably between 1 and 1,000, more preferably between 2 and 500. The PEG has a preferred average molecular weight between 1000 and 40,000, more preferably between 2000 and 20,000, most preferably
10 between 3,000 and 12,000. Preferably, PEG has at least one hydroxy group, more preferably it is a terminal hydroxy group. It is this hydroxy group which is preferably activated to react with a free amino group on the inhibitor. However, it will be understood that the type and amount of the reactive groups may be varied to achieve a covalently conjugated PEG/antibody of the present invention.

15 Water soluble polyoxyethylated polyols are also useful in the present invention. They include polyoxyethylated sorbitol, polyoxyethylated glucose, polyoxyethylated glycerol (POG), etc. POG is preferred. One reason is because the glycerol backbone of polyoxyethylated glycerol is the same backbone occurring naturally in, for example, animals and humans in mono-, di-, triglycerides. Therefore, this branching would
20 not necessarily be seen as a foreign agent in the body. The POG has a preferred molecular weight in the same range as PEG. The structure for POG is shown in Knauf et al., *J. Bio. Chem.* 263:15064-15070 (1988), and a discussion of POG/IL-2 conjugates is found in U.S. Pat. No. 4,766,106, both of which are hereby incorporated by reference in their entireties.

Another drug delivery system for increasing circulatory half-life is the
25 liposome. Methods of preparing liposome delivery systems are discussed in Gabizon et al., *Cancer Research* 42:4734 (1982); Cafiso, *Biochem Biophys Acta* 649:129 (1981); and Szoka, *Ann Rev Biophys Eng* 9:467 (1980). Other drug delivery systems are known in the art and

are described in, e.g., Poznansky et al., *Drug Delivery Systems* (R. L. Juliano, ed., Oxford, N.Y. 1980), pp. 253-315; M. L. Poznansky, *Pharm Revs* 36:277 (1984).

After the liquid pharmaceutical composition is prepared, it is preferably lyophilized to prevent degradation and to preserve sterility. Methods for lyophilizing liquid compositions are known to those of ordinary skill in the art. Just prior to use, the composition may be reconstituted with a sterile diluent (Ringer's solution, distilled water, or sterile saline, for example) which may include additional ingredients. Upon reconstitution, the composition is preferably administered to subjects using those methods that are known to those skilled in the art.

As stated above, the antibodies and compositions of this invention are used to treat human patients to prevent or treat antibody-mediated diseases such as allergies, SLE, PBC and ITP. The preferred route of administration is parenterally. In parenteral administration, the compositions of this invention will be formulated in a unit dosage injectable form such as a solution, suspension or emulsion, in association with a pharmaceutically acceptable parenteral vehicle. Such vehicles are inherently nontoxic and nontherapeutic. Examples of such vehicles are saline, Ringer's solution, dextrose solution, and Hanks' solution. Nonaqueous vehicles such as fixed oils and ethyloleate may also be used. A preferred vehicle is 5% dextrose in saline. The vehicle may contain minor mounts of additives such as substances that enhance isotonicity and chemical stability, including buffers and preservatives.

The dosage and mode of administration will depend on the individual. Generally, the compositions are administered so that antibodies are given at a dose between 1 $\mu\text{g}/\text{kg}$ and 20 mg/kg , more preferably between 20 $\mu\text{g}/\text{kg}$ and 10 mg/kg , most preferably between 1 and 7 mg/kg . Suitably, it is given as an infusion or as a bolus dose, to increase circulating levels by 10-20 fold and for 4-6 hours after the bolus dose. Continuous infusion may also be used after the bolus dose. If so, the antibodies may be infused at a dose between 5 and 20 $\mu\text{g}/\text{kg}/\text{minute}$, more preferably between 7 and 15 $\mu\text{g}/\text{kg}/\text{minute}$. Suitable

treatment regimens are disclosed in WO 00/27428 and WO 00/27433, which are incorporated herein by reference.

Non-Hodgkin's Lymphoma (NHL) originates from components of the spleen, thymus and lymph nodes (Jandl J.H., Non-Hogkin's lymphomas, in Jandl JH (ed): *5 Blood, Textbook of Hematology*, Boston, MA, Little Brown, 1996, pp. 853-887). It consists of a group of lymphocytic malignancies that are derived primarily from B and T cells. Patients with low grade NHL are usually non-responsive to radiation therapy and chemotherapy. This low response rate and the high probability of relapse contribute to the median patient survival time of fewer than 9 years.

10 The imbalance of growth/survival signal by CD40 and crippled death signal by Fas plays an important role in the pathogenesis of low grade B lineage malignancies, including chronic lymphocytic leukemia (CLL) and NHL (Ghia P., *Adv. Cancer Res.*, 2000, 79:157-73). Studies in low grade NHL suggests the disease onset is due to the accumulation of the lymphoma cells as a results of reduction in apoptosis through the Fas 15 pathway and increase in the survival signal through CD40 (Ghia P., *Adv. Cancer Res.*, 2000, 79:157-73). This may explain the insensitivity of the lymphoma cells to chemo or radiation therapy, which specifically target actively proliferating cells.

The invention further relates to a new NHL therapy comprising the use of an antibody to CD40 to block the survival signal for the NHL cells. This strategy is supported 20 by a number of observations in the published scientific literature. CD40 is expressed on the surface of B cells throughout B-cell development. Studies have demonstrated that CD40 provides a survival signal for malignant B cells and stimulates their growth *in vitro* (Romano M.F. et al., *Leuk. Lymphoma*, 2000 Jan., 36(3-4):255-62; Furman R.R., *J. Immunol.*, 2000 Feb. 15, 164(4):2200-6; Kitada S., *Br. J. Haematol.*, 1999 Sep., 25 106(4):995-1004; Romano M.F., *Blood*, 1998 Aug. 1, 92(3):990-5; Jacob A., *Leuk. Res.*, 1998 Apr., 22(4):379-82; Wang D., *Br. J. Haematol.*, 1997 May, 97(2):409-17; Planken E.V., *Leukemia*, 1996 Mar., 10(3):488-93; Greiner A., *Am. J. Pathol.*, 1997 May, 150(5):1583-93). There is evidence from patients that the microenvironment exists to

provide CD40L for CD40 signaling in vivo: CD40 is expressed on lymphoma cells in 86% of patients with B-lineage NHL (Uckun F.M., *Blood*, 1990 Dec. 15, 76(12):2449-56). The discovery of CD40/CD40L co-expression in the same B-cell lymphoma cells raises the possibility of an autocrine growth signal loop in NHL patients (Clodi K., *Br. J. Haematol.*, 1998 Oct., 103(1):270-5). There is also a significant increase in soluble CD40L in the NHL patient serum (Younes A., *Br. J. Haematol.*, 1998 Jan., 100(1):135-41). The soluble CD40L can induce proliferation of lymphoma cells in primary NHL lymphoma cell culture (Andersen N.S., *Blood*, 2000 Sep. 15, 96(6):2219-25; Buske C., *Leukemia*, 1997 Nov., 11(11):1862-7). CD40L expression is increased in the tumor marginal zone in low-grade MALT lymphomas (Carbone A., *Am. J. Pathol.*, 1995 Oct., 147(4):912-22; Greiner A., *Dev. Immunol.*, 1998, 6(3-4):187-95). Given the high level of CD40 expressed on tumors of B-cell lineage and its function as a survival signal for these malignant cells, an antagonist anti-CD40 antibody may have therapeutic value in NHL.

A human IgG2 subtype anti-human CD40 monoclonal antibody generated by immunization of transgenic mice bearing the human IgG2 heavy chain locus and the human K light chain locus is suitable for use. To demonstrate the potential efficacy of 15B8 in a preclinical *in vitro* model of NHL, 15B8 is tested using malignant B cells (NHL cells) obtained from NHL patients who are either rituximab treated or naïve. Rituximab is an anti-CD20 monoclonal antibody for the treatment of relapsed or refractory low grade or follicular NHL.

Since primary lymphoma cells do not proliferate in regular culture medium and undergo apoptosis after a few days in culture, tumor cells are co-cultured with irradiated CD40-ligand (CD40L) transfected feeder cells (Arpin, C., *Science*, 1995, 268:720-722) in the presence or absence of the B cell growth factor Interleukin-4 (IL-4). Antibodies (agonist anti-CD40 MS81, or antagonist anti-CD40 antibody or isotype control huIgG2) of varying concentration (from 0.01 µg/ml to 10 µg/ml) are added to the culture. Following incubation at 37°C for 48 hours, cultured cells are pulsed with ³H-thymidine for 18 hours. The cells are then harvested and analyzed for the amount of ³H-thymidine

incorporation (Schultz, J.L., *Proc. Natl. Acad. Sci. USA*, 1995, 92:8200-8204). Preferably, all sample conditions are in triplicate. In these NHL cell primary culture assays, antibody alone or in combination with IL-4 should not stimulate NHL cells to proliferate *in vitro*. In contrast, an agonist anti-CD40 antibody will induce NHL cell proliferation under the same condition.

There are two types of preclinical models that are currently used for evaluation of human antigen-specific Mabs in therapeutic development for lymphomas. One model is the xenograft mouse *in vivo* model, where the EBV-transformed lymphoma cell lines, such as Daudi (Burkitt lymphoma) or Raji (Burkitt lymphoma) cells, are xenografted into SCID/Nude mice. However, in these models, the results only reflect effects on the particular immortal cell line, which is derived from one EBV-transformed cell. It is known that Burkitt lymphoma cells are lymphoblastoid cells (Ambinder R.F., *Cancer Treat. Res.*, 1999, 99:27-45; Quintanilla-Martinez L., *Leuk. Lymphoma*, 1998 Jun., 30(1-2):111-21; Klein G, *Acta Microbiol. Immunol. Hung.*, 1996, 43(2-3):97-105) while the lymphoma cells from NHL patients are believed to be at the mature B cell stage (Ghia P., *Adv. Cancer Res.*, 2000, 79:157-73). EBV transformation of B cells results in changes of many components in the CD40 signaling pathway (Uchida J., *Science*, 1999 Oct. 8, 286(5438):300-3; Farrell P.J., *Biomed. Pharmacother.*, 1997, 51(6-7):258-67). In contrast to CD40 signaling in NHL cells and normal B cells, CD40 signaling leads to growth arrest in EBV-transformed Burkitt lymphoma cell lines (Fukuda M., *Viral Immunol.*, 2000, 13(2):215-29; Baker M.P., *Blood*, 1998 Oct. 15, 92(8):2830-43). Thus, the results of testing an antagonist anti-CD40 MAb in the xenograft models will not be able to predict the response to the antibody by NHL patients.

The other model is the *in vitro* growth inhibition assay of lymphomas cells from NHL patients, which is preferably used herein. The advantage is that the results predicated the sensitivity of the lymphoma cells from NHL patients to the agent tested. However, the results are obtained from *in vitro* study under defined conditions. A previously published study reported that a rat anti-mouse CD40, which failed to induce

ADCC and CDD *in vitro*, showed good efficacy in two syngeneic mouse B lymphoma models (BCL1 and A31) (Tutt A.L., *J. Immunol.*, 1998 Sep. 15, 161(6):3176-85). The anti-tumor effect on the anti-mouse CD40 occurred slower in time than an anti-Id tested. The anti-mouse CD40 may operate by blocking critical growth signals that are dependent on the expression of surface CD40 not direct signaling like anti-Id in the mouse models tested. This study suggests that the blocking of CD40/CD40L signaling by an anti-CD40 could be efficacious *in vivo*. When tested, the antibody should not bind to the Fc_y receptors *in vitro* and will fail to induce ADCC and CDC *in vitro* since it is of human IgG2 subtype. Anti-CD 40 antibodies having these properties should be beneficial to NHL patients, especially
10 Rituxan-resistant patients.

The present invention will now be illustrated by reference to the following examples which set forth particularly advantageous embodiments. However, it should be noted that these embodiments are illustrative and are not to be construed as restricting the invention in any way.

15

EXAMPLES

GENERAL METHODS

ELISA Assay for Immunoglobulin Quantification

The concentrations of human IgM and IgG are estimated by ELISA. 96-well ELISA plates are coated with 2 µg/ml goat anti-human IgG MAb (The Jackson Laboratory, Bar Harbor, Maine) or with 2 µg/ml goat anti-human IgM MAb 4102 (BioSource International, Calif.) in 0.05M carbonate buffer (pH 9.6), by incubation for 16 hours at 4°C. Plates are washed 3 times with PBS-0.05% Tween-20 (PBS-Tween) and saturated with BSA for 1 hour. After 2 washes the plates are incubated for 2 hour at 37°C with different dilutions of the test samples. After 3 washes, bound Ig is detected by incubation for 2 hour at 37°C with 1 µg/ml peroxidase-labeled goat anti-human IgG MAb or goat anti-human IgM MAb. Plates are washed 4 times and bound peroxidase activity is revealed by the addition of O-

phenylenediamine as a substrate. Human IgG or IgM standards (Caltaq, Burlingame, CA) is used to establish a standard curve for each assay.

Isolation of the Peripheral Blood Mononuclear Cells (PBMC) from Human Peripheral Blood.

5 20ml of Ficoll-Paque solution (low endotoxin, Pharmacia) is added per 50 ml polystyrene tube, in 3 tubes, 30 minutes before adding the blood. The Ficoll-Paque solution is warmed up to room temperature. 3L of bleach in 1:10 dilution is prepared, and used to wash all the tubes and pipettes contacting the blood. The blood is layered on the top of the Ficoll-Paque solution without disturbing the Ficoll layer, at 1.5ml blood/1ml of
10 Ficoll-Paque. The tubes are centrifuged at 1700 rpm for 30 minutes at room temperature with the brake on the centrifuge turned off. As much of the top layer (plasma) as possible is removed, minimizing the vacuum in order to avoid removing the second layer of solution. The second layer, which contains the B and T lymphocytes, is collected using a sterile Pasteur pipette, and place in two 50 ml polystyrene tubes. The collection is diluted
15 with 3x the volume of cold RPMI with no additives, and the tubes are centrifuged at 1000 RPM for 10 minutes. The media is removed by aspiratation, and the cells from both 50 ml tubes are resuspended in a total of 10ml cold RPMI (with additives) and transferred to a 15ml tube. The cells are counted using the hemacytometer, then centrifuged at 1000 RPM for 10 minutes. The media is removed and the cells resuspended in 4mls RPMI. This
20 fraction contained the PBMC.

Isolation of the B cells from PBMC.

100 μ l of Dynabeads (anti-hCD19) are placed in a 5 ml plastic tube. 3ml of sterile PBS are added to the beads and mixed, and placed in the magnetic holder, then allowed to sit for 2 minutes. The solution is removed using a Pasteur pipette. 3mls of
25 sterile PBS are added, mixed and placed in the magnetic holder, then let sit for 2 minutes. This procedure with sterile PBS is repeated one more time for a total of 3 washes. The

PBMC is added into the beads and incubated, while mixing, for 30 minutes at 4°C. The tube containing the PBMC and beads is placed into the magnetic holder for 2 minutes, then the solution is transferred to a new 5ml tube in the magnetic holder. After 2 minutes, the solution is transferred to a new 15ml tube. This step is repeated four more times, and the
5 solutions of the first four times are collected in the 15ml tube, then centrifuged at 1000 RPM for 5 minutes. This step produced the pellet for T cell separation.

100 μ l RPMI (with additives) is added to collect the beads, and the solution is transferred into a 0.7ml tube. 10 μ l of Dynal DetachaBeads are added into the suspension at room temperature, and it is allowed to rotate for 45 minutes. The suspension is
10 transferred into a new 5ml tube and 3mls of RPMI (with additives) is added. The tube is placed in the magnetic holder for 2 minutes. The solution is transferred into a new 5ml tube in the holder for 2 minutes, then to a 15ml tube. The previous step is repeated three more times, collecting the solution in the 15ml tube. The 15ml tube is centrifuged at 1000RPM for 10 minutes, and the cells resuspended in 10ml RMPI. The washing step is
15 repeated 2 more times for a total of 3 washes. The cells are counted before the last centrifugation. This step completed the B cell purification. Cells are stored in 90% FCS and 10% DMSO and frozen at -80°C.

Isolation of the T Cells.

The human T cell Enrichment Column (R&D systems, anti-hCD3 column
20 kit) is prepared using 20ml of 1X column wash buffer by mixing 2ml of 10X column wash buffer and 18ml of sterile distilled water. The column is cleaned with 70% ethanol and placed on top of a 15ml tube. The top cap of the column is removed first to avoid drawing air into the bottom of the column. Next, the bottom cap is removed, and the tip is cleaned with 70% ethanol. The fluid within the column is allowed to drain into the 15ml tube. A
25 new sterile 15ml tube is placed under the column after the column buffer had drained to the level of the white filter. The B cell depleted PBMC fraction is suspended in 1ml of buffer and added it to the top of the column. The cells are allowed to incubate with the column at

room temperature for 10 minutes. The T cells are eluted from the column with 4 aliquots of 2ml each of 1X column wash buffer. The collected T cells are centrifuged at 1000 RPM for 5 minutes. The supernatant is removed and the cells resuspended in 10mls RPMI. Cells are counted and centrifuged one more time. The supernatant is removed, completing 5 the T cell purification. Cells are stored in 90% FCS and 10% DMSO and frozen at -80°C.

For the above procedures, the RPMI composition contains 10 % FCS (inactivated at 56°C for 45 min.), 1% Pen/Strep (100u/ml Penicillin, 0.1µg/ml Streptomycin), 1% Glutamate, 1% sodium puravate, 50µM 2-ME.

Flow Cytofluorometric Assay

10 Ramos cells (10^6 cells/sample) are incubated in 100 µl primary antibody (10 µg/ml in PBS-BSA) for 20 min at 4°C. After 3 washes with PBS-BSA or HBSS-BSA, the cells are incubated in 100 µl FITC-labeled F(ab')₂ fragments of goat anti-(human IgG) antibodies (Caltaq) for 20 min at 4°C. After 3 washes with PBS-BSA and 1 wash with PBS, the cells are resuspended in 0.5 ml PBS. Analyses are performed with a FACSCAN V 15 (Becton Dickinson, San Jose, Calif.).

Generation of Hybridoma Clones

Splenocytes from immunized mice are fused with SP2/0 or P3 x 63Ag8.653 murine myeloma cells at a ratio of 10:1 using 50% polyethylene glycol as previously described by de Boer et al., *J. Immunol. Meth.* 113:143 (1988). The fused cells are 20 resuspended in complete IMDM medium supplemented with hypoxanthine (0.1 mM), aminopterin (0.01 mM), thymidine (0.016 mM) and 0.5 ng/ml hIL-6 (Genzyme, Cambridge, Mass.). The fused cells are then distributed between the wells of 96-well tissue culture plates, so that each well contained 1 growing hybridoma on average.

After 10-14 days the supernatants of the hybridoma populations are 25 screened for specific antibody production. For the screening of specific antibody production by the hybridoma clones, the supernatants from each well are pooled and tested

for anti-CD40 activity specificity by ELISA first. The positives are then used for fluorescent cell staining of EBV-transformed B cells as described for the FACS Assay above. Positive hybridoma cells are cloned twice by limiting dilution in IMDM/FBS containing 0.5 ng/ml hIL-6.

5

EXAMPLE 1

EXPRESSION OF HUMAN CD40 IN SF9 CELLS

Sf9 insect cells infected with recombinant virus *Autographa californica* baculovirus (AcNPV), encoding CD40, are cultured for 48 hours in 24-well plates. After removal of the tissue culture medium the plates are incubated for 45 minutes at room temperature (RT) with 0.25 ml of antibody in PBS with 1% BSA (PBS-BSA). After three washed with PBS-BSA, the plates are incubated for 35 minutes at RT with 250 µl of a 1/250 dilution of goat anti-(mouse total Ig) immunoglobulins conjugated to horseradish peroxidase (Zymed, South San Francisco, Calif.) in PBS-BSA. Unbound peroxidase activity is removed by washing five times with PBS-BSA. Bound peroxidase activity is revealed by the addition of an assay mixture prepared by diluting 0.5 ml of 2 mg/ml 3,3',5,5'-tetramethylbenzidine in ethanol to 10 ml with 10 mM sodium acetate, 10 mM EDTA buffer (pH 5.0) and adding 0.03% (v/v) H₂O₂. The reaction is stopped after 10 minutes by adding 100 µl of 1 M H₂SO₄.

EXAMPLE 2

ANTIBODIES RAISED IN HUMAN IMMUNOGLOBULIN TRANSGENIC MICE

Mice transgenic for the human IgG2 heavy chain locus and the human K light chain locus are immunized with Sf9 cells expressing human CD40. The method of immunization is carried out as described by de Boer, U.S. Patent No. 6,004,552. Briefly, mice are injected intraperitoneally at day 0 and day 14 with 5 x 10⁶ Sf9 cells infected with AcCD40 virus. At day 21, 100 µl of serum is obtained to test for the presence of specific

antibodies. After a rest period of at least two weeks, the mice receive a final injection with 5×10^6 cells infected with AcCD40 virus. Three days after this last injection, the spleen cells are used for cell fusion.

Mice are selected for fusion based on the reactivity of their sera with recombinant CD40 in an ELISA. Spleen cells from immunized mice are fused with mouse myeloma cells (NS/0) by the method of Kohler and Milstein, *Nature* 256:495-96 (1975), with modifications. Hybridomas grown in HAT selective medium are selected for further characterization based on their ability to bind CD40 in an ELISA. Hybridomas that produce antibodies that bound nontransfected Sf9 cell lysate or anti-mouse light chain antibody are dropped from consideration. Hybridomas that produced CD40-binding antibodies that did not bind Sf9 cell lysate or anti-mouse light chain antibody are subcloned. Subclones are used to produce antibodies for further characterization. Hybridoma antibodies are also tested for their ability to stain Ramos lymphoma cells, which express human CD40 on their surfaces.

Hybridoma antibodies are then selected for their ability to inhibit the production of IgM by human peripheral blood B cells stimulated with anti-CD28-activated human peripheral blood T cells. Hybridoma antibodies are further screened for their ability to inhibit proliferation of human peripheral blood B cells induced by CD40L and anti-IgM. Hybridomas are also screened for their ability to induce proliferation in resting human peripheral blood B cells. Thus, even when able to bind CD40, not all human antibodies may exhibit the desired inhibitory effect.

EXAMPLE 3

BINDING PROPERTIES OF SELECTED HYBRIDOMAS

Hybridomas are selected based on their inhibitory effect on B cell activation as described above in Example 2. Their binding properties are determined by BIACore evaluation using soluble recombinant CD40 as the mobile phase with the anti-CD40 antibodies being captured on the sensor surface. Inhibitory antibodies may exhibit binding

affinities suitable for the uses described herein in the ranges: K_a (M-1s-1) $1.3 \times 10^5 - 2.4 \times 10^6$; $K_d/\text{sec} = 1.0 \times 10^{-1} - 4.1 \times 10^{-3}$; K_D (M) $1.7 \times 10^{-7} - 3.1 \times 10^{-9}$.

EXAMPLE 4 VARIABLE HYBRIDOMA BINDING TO CD40

5 The relative binding properties of the selected monoclonal antibodies are examined by flow cytometry. Whole blood is incubated for 30 minutes with various concentrations of unlabeled hybridoma antibody plus 0.1 μg of FITC-conjugated 15B8 and anti-CD20-PECy5. The RBC's are then lysed, the leukocyte populations fixed, and the preparations acquired for analysis.

10 The results should show the ability of antibodies of the invention to compete with each other for binding to epitopes. The labeled MAb's are also used to stain peripheral blood cells from humans, Rhesus (*Macaca mulatta*), and cynomolgus (*Macaca fascicularis*) macaques.

EXAMPLE 5 INHIBITION OF IMMUNOGLOBULIN SECRETION BY HUMAN PERIPHERAL B CELLS

15 Plates are coated with anti-human CD3 (2 $\mu\text{g}/\text{ml}$, UCHTi, NE/LE, Pharmingen), at 4°C overnight. The pre-coated plates are washed 3 x with PBS. The T cells are irradiated with 3000 Rad. The B cells are resuspended in RPMI(+) to 10^4 per ml. 100 μl of B cells are added into the well, then anti-CD40 antibodies are added into the well.

20 The T cells are resuspended in RPMI(+) to 10^5 per ml. Human recombinant IL2 to 200u/ml is added (Chiron, 10u/ μl water solution stored at -20°C) in the cell suspension. A 100 μl suspension is taken into each well, and mixed well with the B cells and antibodies. RPMI(+) is added to the wells to a total of 200 μl . The plates are incubated at 37°C for 8 days before harvesting the media, after spinning down the cells for ELISA.

The results should show that in the presence of an antibody according to the invention, the secretion of immunoglobulin, IgM, by T cell-stimulated human peripheral blood B cells, is decreased.

EXAMPLE 6

5 INHIBITION OF PROLIFERATION OF JURKAT-STIMULATED HUMAN PERIPHERAL B CELLS

B cells are purified as described above. 10^4 purified B cells, 10^5 irradiated Jurkat cells (3000 Rad), and antibodies to be tested are added into anti-CD3-coated 96-well plates. The plates are incubated at 37°C for four days, with labeling of the cells with ^3H -thymidine during the last 18 hours. The cells are harvested and counted.

10 The results should indicate that in the presence of the antibodies of the invention, B cell proliferation stimulated by Jurkat cells is inhibited.

EXAMPLE 7

INHIBITION OF PROLIFERATION OF CD40L-STIMULATED HUMAN PERIPHERAL B CELLS

B cells are purified as described above. 10^4 purified B cells, 2×10^4
15 Formaldehyde-fixed CHO-CD40L cells, and antibodies to be tested are added into anti-CD3-coated 96-well plates. The plates are incubated at 37°C for four days, with labeling of the cells with ^3H -thymidine during the last 18 hours. The cells are harvested and counted.

EXAMPLE 8

20 STIMULATION OF B CELL PROLIFERATION

B cells (1×10^4 per well) are cultured in 200 µl RPMI supplemented with 10% fetal calf serum in U-bottom 96-well microtiter plates. B cells are stimulated by addition of immobilized anti-(IgM) antibodies (5 µg/ml, Sigma). Varying concentrations of

MAbs are added at the onset of the microcultures and proliferation is assessed at day 4 by measurement of the incorporation of ^3H -thymidine after 18 hour pulsing.

EXAMPLE 9

EFFECT OF ANTI-CD40 ANTIBODIES ON PROLIFERATION OF HUMAN PERIPHERAL B CELLS 5 STIMULATED BY ANTI-IGM

10^4 purified B cells, anti-IgM beads (5 $\mu\text{g/ml}$, Sigma), and antibodies to be tested are added into 96 well plates. The plates are incubated at 37°C for four days, with labeling of the cells with ^3H -thymidine during the last 18 hours. The cells are harvested and counted.

10

EXAMPLE 10

EFFECT OF ANTI-CD40 ANTIBODY CROSSLINKING ON PROLIFERATION OF HUMAN PERIPHERAL B CELLS

10^4 purified B cells are added into anti-CD40-coated 96 well plates. The plates are incubated at 37°C for four days, with labeling of the cells with ^3H -thymidine 15 during the last 18 hours.

EXAMPLE 11

POLYNUCLEOTIDE AND AMINO ACID SEQUENCES OF HUMAN ANTI-CD40 ANTIBODIES

mRNA is prepared from the hybridomas generated as described in Example 2 and RT-PCR is performed on the mRNAs. Two sets of primers are used to generate PCR 20 products: a universal or pool of heavy and light chain family primers; and then family-specific primers. The PCR products are analyzed on gels, sequenced, and translated.

Sequence information can be used to design additional monoclonal antibodies for use according to the invention. These monoclonal antibodies may differ from those described herein, by substitution of one or more of the framework or CDR 25 regions. The monoclonal antibodies also may differ by substitution of one or more amino

acids, which are shown to differ in certain regions of the framework and CDR. Once the amino acid sequence is designed, routine procedures can be used to construct a corresponding polynucleotide sequence for expression of the monoclonal antibody. Expression and purification of the monoclonal antibodies is performed using methods known in the art, such as those disclosed in U. S. Patent Nos. 5,545,403, 5,545,405, and 5,998,144, which are incorporated herein by reference.

EXAMPLE 12

EFFECT OF 15B8 ON MALIGNANT B-CELL PROLIFERATION *IN VITRO*

To test if 15B8 provides the growth signal like CD40L *in vitro*, B cells from tumor infiltrated lymph nodes (NHL cells) are obtained from one antibody naïve, one rituximab-sensitive and one rituximab-resistant NHL patient. The NHL cells are studied under four different culture conditions; no added antibody (medium); addition of human isotype antibody IgG2 (control); addition of one anti-CD40 antibody (agonistic antibody); and addition of a preferred anti-CD40 antibody (non-agonistic antibody). All antibodies are tested 1, 2, and 5 µg/mL in the presence or absence of IL-4. The NHL cells from two patients are cultured as described above under the same four conditions in the presence of IL-4 (2 ng/ml). B-cell proliferation is measured by ^3H -thymidine incorporation as described above.

Non-agonistic anti-CD40 antibody, at concentration of 1, 2 and 5 µg/mL, should not stimulate NHL cells to proliferate in either the presence or absence of IL-4. In contrast, an agonistic anti-CD40 antibody, tested at the same concentration, will stimulate NHL-cell proliferation in the presence and absence of IL-4 in all patient samples. Results from the NHL cells from the two patients in the presence of IL-4 and three patients in the absence of IL-4 are comparable. These results should indicate that a non-agonistic anti-CD40 antibody will not stimulate proliferation of NHL cells from rituximab-sensitive, naïve or -resistant NHL patients *in vitro*.

FACS analysis of the NHL cells is performed with either a direct labelled antibody or antibody plus anti-HuIgG2-FITC to confirm that CD40 is expressed on the surface the NHL cells tested and that the antibody binds to the NHL cells. The NHL cells from two rituximab-sensitive and four rituximab-resistant patients were tested. NHL cells 5 from all the patients expressed CD40 and bound to 15B8. The antibody binding-positive cell population in any given patient is expected to be about 60% to 90%.

EXAMPLE 13

15B8 INHIBITS CD40L-STIMULATED PROLIFERATION OF NHL CELLS *IN VITRO*

To evaluate the ability of non-agonistic anti-CD40 antibody to block the 10 growth signal provided by CD40L *in vitro*, NHL cells from patients are cultured as described in Example 12 in suspension over CD40L-expressing feeder cells under four different conditions: no added antibody (medium); addition of human isotype antibody IgG2 (control); addition of anti-CD40 antibody (agonistic antibody); and addition of non-agonistic anti-CD40 antibody. All antibodies are added at concentration of 1, 2, and 5 15 µg/mL in the presence or the absence of IL-4. The NHL cells from one antibody naïve, two rituximab-sensitive and five rituximab-resistant patients are cultured under the same four conditions as described above in the presence of IL-4 (2 ng/ml). NHL cells from three rituximab-sensitive and four rituximab-resistant patients are cultured under similar 20 conditions in the absence of IL-4. The NHL cell proliferation is measured by ^3H -thymidine incorporation.

Non-agonistic anti-CD40 antibody is expected to inhibit the proliferation by about 10-70% when compared to the control. The degree of inhibition by this antibody varies depending on patient samples and the dose level of antibody. There should be a statistically significant dose response ($p<0.005$), the inhibitory effect increases with 25 increasing antibody dose.

EXAMPLE 14

TESTING AGONISTIC AND ANATAGONISTIC ACTIVITY OF NON-AGONISTIC ANTI-CD40
ANTIBODY IN DIFFERENT SPECIES *IN VITRO*

5 To determine if it is an agonist or antagonist anti-CD40, non-agonistic anti-CD40 antibody is tested in several *in vitro* assays described below using cells from humans and five different primate species, including chimpanzee (chimp), marmoset, cynomologus monkey, rhesus monkey and baboon.

Non-agonistic anti-CD40 antibody does not activate human peripheral blood

10 B cell and does not cause PBMC proliferation *in vitro* in human, chimp and marmoset. Activation of CD40 on human B cells obtained from peripheral blood leads to proliferation of the B cells (van Kooten C., *J. Leukoc. Biol.*, 2000 Jan., 67(1):2-17; Denton M.D., *Pediatr. Transplant.*, 1998 Feb., 2(1):6-15; Evans D.E., *J. Immunol.*, 2000 Jan. 15, 164(2):688-97; Noelle R.J., *Agents Actions Suppl.*, 1998, 49:17-22; Lederman S. et al.,
15 *Curr. Opin. Hematol.*, 1996, 3(1):77-86). To test if non-agonistic anti-CD40 antibody activates CD40 on B cells, a series of proliferation assays is carried out using freshly isolated human B cells or PBMCs from peripheral blood. The effect of non-agonistic anti-CD40 antibody in this assay is measured by ^3H methyl-thymidine incorporation (John E. Coligan et al., *Current Protocols in Immunology*, Vol. 13:12, John Wiley & Sons, Inc.,
20 1991; Kwekkeboom J., *Immunology*, 1993 Jul, 79(3):439-444). At concentrations of 0.2, 1 and 5 $\mu\text{g}/\text{ml}$, non-agonistic anti-CD40 antibody is expected to have minimal effect on purified B cell proliferation compared to the effect on CD40L, which demonstrates strong proliferation-promoting effect on human B cells.

25 Non-agonistic anti-CD40 antibody is further compared to CD40L for stimulation of human PBMC proliferation using freshly isolated human PBMC. Non-agonistic anti-CD40 antibody is not expected to stimulate human PBMC proliferation *in vitro* as measured by ^3H methyl-thymidine incorporation (John E. Coligan et al., *Current*

Protocols in Immunology, Vol. 13:12, John Wiley & Sons, Inc., 1991; Kwekkeboom J., *Immunology*, 1993 Jul, 79(3):439-444) at a concentration range of 0.2-5 μ g/ml.

Upon B cell activation, a number of cell surface proteins are up-regulated (Denton M.D., *Pediatr. Transplant.*, 1998 Feb., 2(1):6-15; Evans D.E., *J. Immunol.*, 2000

5 Jan. 15, 164(2):688-97; Noelle R.J., *Agents Actions Suppl.*, 1998, 49:17-22; Lederman S. et al., *Curr. Opin. Hematol.*, 1996, 3(1):77-86). To confirm that non-agonistic anti-CD40 antibody does not activate human B cells and does not induce an agonist signal when bound to CD40, its ability to up-regulate B cell activation markers is tested by FACS analysis using purified human PBMC. There should be no up-regulation in the expression 10 of activation markers such as CD25, CD69, CD86, HLA-DR and ICAM-1 (CD54) in non-agonistic anti-CD40 antibody treated human B cells. The level of these markers should be similar when cells are treated with either non-agonistic anti-CD40 antibody or huIgG2 control. In contrast, CD69 should be consistently up-regulated by CD40L in PBMC samples from three healthy volunteers tested.

15 Additional consequences of B cell activation are up-regulation of surface FasL and apoptosis (Revy P., *Eur. J. Immunol.*, 1998 Nov., 28(11):3648-3654; Carey G.B., *Immunol. Rev.*, 2000 Aug., 176:105-115; Ju S.T., *Int. Rev. Immunol.*, 1999, 18(5-6):485-513; Baumgarth N., *Immunol. Rev.*, 2000 Aug., 176:171-180). To confirm that non-agonistic anti-CD40 antibody is not an agonistic anti-CD40 antibody, its ability to 20 induce FasL expression and apoptosis of human B cells is also tested. Annexin V staining on the cell surface can be used as an early apoptosis marker (Ju S.T., *Int. Rev. Immunol.*, 1999, 18(5-6):485-513). Human B cells are purified from peripheral blood and incubated with non-agonistic anti-CD40 antibody. FACS analysis is used to detect cells with positive staining of Annexin V and anti-FasL. There should be no significant difference on the 25 surface staining by the two reagents between cells incubated with non-agonistic anti-CD40 antibody or the isotype control (huIgG2) antibody.

Non-agonistic anti-CD40 antibody is expected to cross-react with CD40 expressed on the surface of CD20 positive PBMCs from primates. To test if non-agonistic

anti-CD40 antibody can activate CD40 on B cells from other primate species such as chimps and marmosets, the same proliferation assays are carried out using freshly isolated chimp and marmoset PBMC from fifteen chimps and five marmosets. Similar to the results with the human PBMC, non-agonistic anti-CD40 antibody is not expected to stimulate the proliferation *in vitro* of PBMCs from six chimps and five marmosets at 1 and 5 µg/ml concentration. Non-agonistic anti-CD40 antibody also is not expected to up-regulate the expression of activation marker, CD69, in chimp-PBMC samples. Non-agonistic anti-CD40 antibody is not expected to show any effect on FasL expression and apoptosis in chimp PBMCs similar to human PBMC controls after 24 and 48 hours simulation *in vitro* in samples from chimps.

Cross-linking non-agonistic anti-CD40 antibody by a secondary antibody fixed to plastic surface should not increase its potency to stimulate B cell proliferation. When tested using PBMCs from humans and chimps in this cross-linking assay, non-agonistic anti-CD40 antibody should not stimulate proliferation of the cells.

In summary, non-agonistic anti-CD40 antibody should not initiate an activation signal in human B cells/PBMCs nor in chimp/marmoset PBMCs *in vitro*.

EXAMPLE 15

TESTING ANTAGONIST ACTIVITY OF ANTI-CD40 ANTIBODY IN HUMANS, CHIMPS AND MARMOSETS *IN VITRO*.

To determine if non-agonistic anti-CD40 antibody is an antagonist anti-CD40, its ability to inhibit CD40-CD40L interaction is tested in a CD40L mediated-human B cell proliferation assay (Kwekkeboom J., *Immunology*, 1993 Jul, 79(3):439-444). A transfected CHO cell line expressing human CD40L is used to stimulate the proliferation of purified human peripheral blood B cells or PBMCs. Human B cells from healthy volunteers and human PBMCs from healthy volunteers are tested. In all the samples tested, non-agonistic anti-CD40 antibody is expected to suppress CD40L-expressing CHO cells mediated-proliferation by 40-90% at concentration range from 0.2 - 5µg/ml. The no-effect

dose of non-agonistic anti-CD40 antibody is expected to be 0.008 µg/ml and is expected to reach a saturating dose at 0.2 µg/ml.

Additional assays are carried out using freshly isolated PBMCs from chimps and marmosets. As with the human PBMCs, non-agonistic anti-CD40 antibody is expected 5 to inhibit the proliferation of chimp and marmoset PBMCs stimulated by CD40L expressing-CHO cells at 1µg/ml concentration level. The inhibition by non-agonistic anti-CD40 antibody is expected to be approximately 55-75% and 65-85% in PBMC samples from chimps and marmosets respectively.

Activated B cells undergo a number of biological response such as 10 proliferation and antibody production. The activation of B cells by T cell-dependent antigens involves CD4⁺ T-helper (Th) cells. This T cell helper process is mediated by a concerted effort of the interaction of CD40 on the B cells with the CD40L on the Th cells surface together with the interactions of other co-stimulatory factors and cytokines Denton M.D., *Pediatr. Transplant.*, 1998 Feb., 2(1):6-15; Evans D.E., *J. Immunol.*, 2000 Jan. 15, 15 164(2):688-97; Noelle R.J., *Agents Actions Suppl.*, 1998, 49:17-22; Lederman S. et al., *Curr. Opin. Hematol.*, 1996, 3(1):77-86; Mackey M.F., et al., *J. Leukoc. Biol.*, 1998, 63(4):418-428). To test if non-agonistic anti-CD40 antibody can block T-helper cell mediated B cell antibody production, purified human peripheral blood B cells are cultured in the presence of purified irradiate T cells activated with anti-CD3 antibody. An ELISA 20 assay is used to measure the level of IgM production. Non-agonistic anti-CD40 antibody is expected to reduce IgM production by about 30% in this assay. Therefore, non-agonistic anti-CD40 antibody should reduce T cell-mediated B cell immunoglobulin production.

EXAMPLE 16

TESTING AGONIST ANTI-MONKEY (CYNOMOLGUS, RHESUS AND BABOON) ACTIVITY OF CD40

25 ANTIBODY *IN VITRO*.

FACS analysis should demonstrate that non-agonistic anti-CD40 antibody binds to CD40 expressed on the surface of B cells from peripheral blood of monkeys

(rhesus, cynomologus and baboon). The effect of non-agonistic anti-CD40 antibody on freshly isolated cynomolgus monkey PBMC is tested in the same proliferation assay described above for human and chimps (John E. Coligan et al., *Current Protocols in Immunology*, Vol. 13:12, John Wiley & Sons, Inc., 1991; Kwekkeboom J., *Immunology*, 5 1993 Jul, 79(3):439-444). In contrast to human PBMC, non-agonistic anti-CD40 antibody is expected to stimulate cynomolgus monkey PBMC to proliferate *in vitro* as measured by ³H methyl-thymidine incorporation. At 1 µg/ml level, non-agonistic anti-CD40 antibody is expected to stimulate the proliferation of the PBMCs by about 5 - 130 folds compare to the huIgG2 control in samples from monkeys. At 5µg/ml level, the proliferation expected to be 10 stimulated by non-agonistic anti-CD40 antibody is 14 - 24 folds in samples from some monkeys and about 1.3 or 1.9 fold in samples from other monkeys. This suggests that, at concentration level of 5µg/ml, non-agonistic anti-CD40 antibody may be at the limit of over-saturating dose for its proliferation-stimulatory effect on PBMCs from cynomolgus 15 monkey. Further FACS analysis of B cells for activation status by surface markers is expected to indicate that non-agonistic anti-CD40 antibody induces CD69, CD86 and HLA-DR up-regulation on monkey B cells.

To determine whether this agonistic effect of non-agonistic anti-CD40 antibody is not cynomolgus monkey specific, the same assays are performed using PBMCs from rhesus monkeys and baboons. Similar results to that obtained from cells of 20 cynomolgus monkeys are expected. Non-agonistic anti-CD40 antibody is expected to stimulate proliferation of PBMCs from rhesus monkeys and baboons *in vitro*.

EXAMPLE 17

TESTING AGONIST ACTIVITY OF ANTI CD40 ANTIBODY *IN VIVO* IN CYNOMOLGUS MONKEYS.

Non-agonistic anti-CD40 antibody is expected to stimulate proliferation and 25 up-regulation of cell surface activation markers in PBMCs from cynomolgus monkeys *in vitro*. To determine if non-agonistic anti-CD40 antibody is an agonist anti-CD40 antibody in the monkeys *in vivo*, a study is performed to examine the biodistribution of non-

agonistic anti-CD40 antibody and the fate of affected peripheral B cells (i.e. extravasation, apoptosis, activation status, or complement lysis) [Biodistribution 15B8.72 Antibodies following Intravenous Administration to Non-Naïve Male and Female Cynomolgus Monkeys (SNBL.218.3, SNBL USA)].

5 Cynomolgus monkeys receive a single intravenous administration of 3 mg/kg non-agonistic anti-CD40 antibody. The following parameters are monitored: clinical signs, food consumption, body weight, pharmacokinetics, serum complement (CH50), flow cytometry for B cells (including apoptotic B cells), T cells, and monocytes. B cell CD40 receptor saturation with non-agonistic anti-CD40 antibody is also measured.

10 Animals are necropsied 24 hours after receiving the single dose of non-agonistic anti-CD40 antibody, and standard organs are weighed. Pre-study surgical biopsies of spleen and axillary lymph nodes are taken to serve as baseline controls. At necropsy, lymphoid and non-lymphoid tissues are sampled for histopathology and immunohistochemistry. Tissues are immunostained with antibodies against CD3, CD40, CD20, CD27, and CD38 antigens.

15 All animals are expected to survive to the scheduled necropsy and there should be no effects on food consumption, body weight, CH50 levels nor on peripheral blood T cell or monocyte counts. There should be no changes in organ weights. Microscopic examination of the spleen is expected to show moderate diffuse follicular hyperplasia with necrosis and/or neutrophilic infiltrates in the germinal centers of all non-

20 agonistic anti-CD40 antibody-treated animals. Examination of mesenteric and inguinal lymph nodes may show mild follicular hyperplasia in 2/3 animals. No treatment related microscopic effects are expected in other tissues (liver, skin, brain, thyroid, lung, bone marrow, adrenal gland and kidney).

Immunostaining with CD20, CD27, CD40 and CD86 antibodies should reveal increases in these markers in splenic and lymph node follicles, which correlate with the follicular hyperplasia expected in these same tissues. Increased staining of CD20 and CD40 are expected to be limited to the spleen and lymph node while there may be some additional staining of hepatic tissue with CD27 and of hepatic Kupffer cells and

inflammatory cells by CD86. CD86 staining may also be increased in thymic medullary cells and adrenal interstitial leukocytes. There should be no changes in the immunostaining of CD3 in non-agonistic anti-CD40 antibody-treated animals as compared to controls.

These experiments should indicate that a single dose of 3 mg/kg of non-agonistic anti-CD40 antibody administered to cynomolgus monkey can cause proliferation of lymphoid follicles and/or redistribution of B cells from the peripheral blood in spleen and lymph nodes within a 24 hour period. Antibodies to CD20, CD27, CD40 and CD86 recognize antigens expressed on B cells and/or activated B cells, along with recognition of other cell types. Increased numbers of cells expressing these antigens are seen in the spleen and lymph nodes of treated animals, which suggests an increase in the number of activated CD20+ B cells. This study should suggest that non-agonistic anti-CD40 antibody is an agonist anti-CD40 antibody in cynomolgus monkey *in vivo*. The results obtained *in vivo* and *in vitro* are expected to be consistent in cynomolgus monkeys.

Non-agonistic anti-CD40 antibody should have the potential to modify B cell malignancies, such as non-Hodgkin's lymphoma (NHL), where the CD40/CD40L pathway may play a role in the pathogenesis of the diseases.

The present invention has been described with reference to specific embodiments. However, this application is intended to cover those changes and substitutions which may be made by those skilled in the art without departing from the spirit and the scope of the appended claims.

CLAIMS

1. A human monoclonal antibody or fragment thereof that is capable of specifically binding to a human CD40 antigen expressed on the surface of a normal human CD40-expressing cell, said monoclonal antibody or fragment being free of significant agonistic activity, whereby, when said monoclonal antibody or fragment binds to the CD40 antigen expressed on the surface of said normal cell, the growth or differentiation of said normal cell is inhibited.
2. The monoclonal antibody of claim 1, wherein said cell is a B cell, and wherein said monoclonal antibody binds to said human B cell with an affinity (K_D) selected from the group consisting of at least 10^{-5} M, at least 10^{-7} M, at least 10^{-9} M, and at least 10^{-11} M.
3. The fragment of claim 1, wherein said fragment is a member selected from the group consisting of an Fab' fragment, an F(ab)₂ fragment, an Fab fragment, and an F_v fragment of said monoclonal antibody.
4. A human monoclonal antibody or fragment thereof that is capable of specifically binding to a human CD40 antigen expressed on the surface of a normal human B cell, said monoclonal antibody or fragment being free of significant agonistic activity, whereby, when said monoclonal antibody or fragment binds to the CD40 antigen expressed on the surface of said normal B cell, the growth or differentiation of said normal B cell is inhibited.
5. A hybridoma capable of producing a human monoclonal antibody having specificity for a human CD40 antigen expressed on the surface of a normal human B cell, whereby said monoclonal antibody is free of significant agonistic activity, whereby

when said monoclonal antibody binds to the CD40 antigen expressed on the surface of said normal B cell, the growth or differentiation of said normal B cell is inhibited.

6. A method for inhibiting growth or differentiation of a normal human B cell, said method comprising contacting said B cell with an effective amount of a human anti-CD40 monoclonal antibody, said antibody being free of significant agonistic activity, whereby when said antibody binds to said CD40 antigen on said B cell, the growth or differentiation of said B cell is inhibited.

7. The method of claim 6, wherein said monoclonal antibody binds to said human B cell with an affinity (K_D) selected from the group consisting of at least 10^{-5} M, at least 10^{-7} M, at least 10^{-9} M, and at least 10^{-11} M.

8. The method of claim 6, wherein said monoclonal antibody is an Fab' fragment, an $F(ab)_2$ fragment, an Fab fragment, or an F_v fragment of said monoclonal antibody.

9. A method for inhibiting proliferation of a normal human B cell, wherein said proliferation is augmented by the interaction of a CD40 ligand with a CD40 antigen expressed on the surface of a B cell, said method comprising contacting said B cell with an effective amount of a human anti-CD40 monoclonal antibody, said antibody being free of significant agonistic activity, whereby when said antibody binds to said CD40 antigen on said B cell, the proliferation of said B cell is inhibited.

10. The method of claim 33, wherein said monoclonal antibody binds to said human B cell with an affinity (K_D) selected from the group consisting of at least 10^{-5} M, at least 10^{-7} M, at least 10^{-9} M, and at least 10^{-11} M.

11. A method for inhibiting antibody production by B cells in a human patient, said method comprising administering to a human patient an effective amount of a human anti-CD40 monoclonal antibody, said antibody being free of significant agonistic activity, whereby when said antibody binds to said CD40 antigen on said B cells, antibody production by said B cells is inhibited.

12. The method of claim 11, wherein said monoclonal antibody binds to said human B cell with an affinity (K_D) selected from the group consisting of at least 10^{-5} M, at least 10^{-7} M, at least 10^{-9} M, and at least 10^{-11} M.

13. A method of inhibiting growth of cancer cells of B cell lineage, said method comprising contacting said cancer cells with an effective amount of a human anti-CD40 monoclonal antibody, said antibody being free of significant agonistic activity, whereby when said antibody binds to said CD40 antigen on said cancer cells, the proliferation of said cancer cells is inhibited.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 01/30849

A. CLASSIFICATION OF SUBJECT MATTER		
IPC 7 C07K16/28 C12N5/20 A61K39/395 A61P37/06 A61P35/00		
<p>According to International Patent Classification (IPC) or to both national classification and IPC</p>		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 7 C07K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the International search (name of data base and, where practical, search terms used) BIOSIS, EMBASE, WPI Data, PAJ, EPO-Internal		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>P. KARLSSON ET AL.: "Selection of human single chain antibodies against CD40." IMMUNOLOGY LETTERS, vol. 73, no. 2-3, September 2000 (2000-09), page 161 XP001073504 Amsterdam, The Netherlands abstract 358</p> <p>---</p> <p>EP 0 945 465 A (CHIRON CORPORATION) 29 September 1999 (1999-09-29) examples claims</p> <p>---</p> <p style="text-align: center;">-/-</p>	1-13
A		1-13
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C.		<input checked="" type="checkbox"/> Patent family members are listed in annex.
<p>* Special categories of cited documents :</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p>		
Date of the actual completion of the International search	Date of mailing of the International search report	
17 April 2002	03/05/2002	
Name and mailing address of the ISA		
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel: (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016		
Authorized officer		
Nooij, F		

INTERNATIONAL SEARCH REPORT

In Application No.
PCT/US 01/30849

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	J. KWEKKEBOOM ET AL.: "CD40 plays an essential role in the activation of human B cells by murine EL4B5 cells." IMMUNOLOGY, vol. 79, no. 3, July 1993 (1993-07), pages 439-444, XP002029781 Oxford, GB cited in the application the whole document	1-13
A	WO 96 34096 A (CELL GENESYS, INC.) 31 October 1996 (1996-10-31) claims 1,14,17,18,24,25	1-13
T	WO 01 83755 A (GEMINI SCIENCE, INC.) 8 November 2001 (2001-11-08) example 3 claims	1-13

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Although claims 6-10 and 13 (all partially, as far as an in vivo method is concerned) and claims 11 and 12 (both completely) are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

INTERNATIONAL SEARCH REPORT

International Application No
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